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Inhibition of Acid Secretion from Parietal Cells by Non-Human-Infecting *Helicobacter* Species: a Factor in Colonization of Gastric Mucosa?

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***Helicobacter pylori* has been shown to produce a protein that inhibits acid secretion from parietal cells. We have examined other non-human-infecting *Helicobacter* species for this property by measuring the uptake of [^{14}C]aminopyrine into rabbit parietal cells as an indirect assessment of acid secretion. *Helicobacter felis* and an isolate from a rhesus monkey were shown to inhibit acid secretion. Isolates of *Helicobacter mustelae* gave variable responses. Whole bacteria and cell-free sonicates impaired the uptake of [^{14}C]aminopyrine. We also tested other bacteria, including *Escherichia coli*, *Proteus vulgaris*, *Klebsiella oxytoca*, and *Campylobacter jejuni*. As whole organisms, these control bacteria had little effect on acid secretion, but sonicates caused pronounced inhibition that was partially heat labile. Pronase treatment of *H. pylori* destroyed its inhibitory effect. These results suggest that most *Helicobacter* species, but not all isolates, are able to inhibit acid secretion from rabbit parietal cells. This property may be a factor in the establishment of long-term infection by these species.**

The bacterium *Helicobacter pylori* is now established as a major causative agent of type B gastritis in humans and is strongly implicated in duodenal ulceration (2). One of the features of acute infection with this bacterium is a period of hypochlorhydria of varying duration. Thus, in the study of a volunteer by Morris and Nicholson (14), the gastric pH rose to 6.8 8 days after ingestion of a pure culture of *H. pylori*. The onset of hypochlorhydria coincided with infection of the fundus of the stomach with the organism and the associated acute inflammation. In a study of young human volunteers (1), two of six naturally infected subjects were found to be hypochlorhydric. A possible explanation for the hypochlorhydria has come from recent studies in this laboratory which have shown that pure cultures of *H. pylori* had antisecretory activity as measured by an in vitro assay of acid secretion, by using suspensions of rabbit parietal cells (3). A bacterial protein was postulated to inhibit parietal cell activity. Thus, the high pHs observed in some *H. pylori*-infected persons may be due to an actual turning off of acid production rather than a disturbance in hydrogen ion diffusion as previously proposed (8). This antisecretory activity could also explain another observation concerning some *H. pylori*-infected patients, that is, the demonstration of the bacteria within the canaliculi of parietal cells (4). The pH within canaliculi is estimated to be less than 2; thus, the organism would not be expected to survive in this environment, at least on the basis of its in vitro pH sensitivity. The stomachs of many other animal species are colonized with spiral-shaped bacteria distinct from *H. pylori*. These *Helicobacter* species tend to colonize in much higher numbers than *H. pylori*, and the observation of organisms within the parietal cell canaliculus is more common. If antisecretory activity is a significant factor in gastric colonization and is the reason bacteria can survive within parietal cells, then these *Helicobacter* species should also switch off acid secretion.

The isolation by one of us (A.L.) of a gastric spirillum (now named *Helicobacter felis*) from the cat (12, 15), which is similar to an organism also seen within the parietal cells of dogs (9), and the availability of *Helicobacter mustelae* from ferrets with gastritis (5) allowed us to test the hypothesis that *Helicobacter* species possess antisecretory activity. We also questioned whether this property is an exclusive feature of *H. pylori* infection. Because we have recently demonstrated that *H. felis* causes acute gastritis in gnotobiotic rats and mice, we wished to ascertain whether *H. felis* also colonizes numerous parietal cells (6, 11).

MATERIALS AND METHODS

Bacterial cultures. The following strains of bacteria were used in the parietal cell assays: three strains of *H. felis* (an American Type Culture Collection strain and two others), a clinical strain of *H. pylori* (H.win), *H. mustelae* strains (an American Type Culture Collection strain plus four other isolates), and an isolate from a rhesus monkey, kindly provided by Andre DuBois, Walter Reed Army Hospital, Bethesda, Md. The *Helicobacter* species used in this study were all laboratory-adapted strains and have all undergone multiple passes. An isolate of *Campylobacter jejuni* (T18) and clinical strains of *Escherichia coli*, *Proteus vulgaris*, and *Klebsiella oxytoca* were also assayed.

Bacterial cell suspensions. *H. pylori*, *H. mustelae*, *H. felis*, a *Helicobacter*-like isolate from a rhesus monkey, and *C. jejuni* were grown on *Campylobacter*-selective agar plates (Skirrow's medium) for 2 to 3 days at 37°C and subsequently on blood agar alone in a microaerobic environment.

E. coli, *P. vulgaris*, and *K. oxytoca* were grown on sheep blood agar for 24 h at 37°C. Cells were harvested in sterile physiological saline by using cotton wool swabs and were washed twice in saline.

The concentration of the organisms was adjusted to an optical density at 600 nm of 1.00 in saline. *Helicobacter* species, as whole organisms, were used in an enriched

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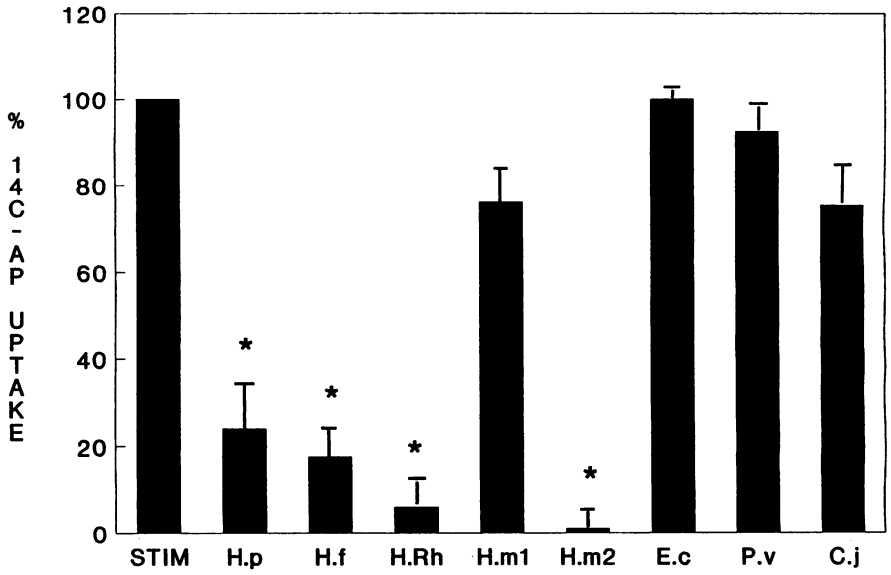

FIG. 1. Effect of whole bacteria on the uptake of [^{14}C]AP by rabbit parietal cells. Abbreviations: STIM, stimulated; H.p, *H. pylori*; H.f, *H. felis*; H.Rh, isolate from a rhesus monkey; H.m1, *H. mustelae* (mean for three noninhibitory strains); H.m2, *H. mustelae* (mean for two inhibitory strains); E.c, *E. coli*; P.v, *P. vulgaris*; C.j, *C. jejuni*. Standard errors are shown as vertical lines from the mean. Asterisks represent statistical significance.

oxygen environment and were unlikely to replicate during the experiment. The whole control organisms, except *C. jejuni*, may have replicated, but this was not quantitated.

In thermal experiments, aliquots of the above suspensions were heated to 60°C for 30 min in a shaking water bath. For sonicates, similar suspensions were sonicated on ice at a setting of 7 with a microtip (cell disrupter W-220F; Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) in four consecutive 30-s treatments.

In vitro assay of parietal cell activity. [^{14}C]AP accumulation. The assay method previously described was used (3). In brief, a rabbit stomach was washed and the body and fundus were cut up into small pieces in Hanks tissue culture fluid and then treated with collagenase at 37°C. Three successive 30-min treatments provided an excellent yield of small glands and single epithelial cells. Aliquots of these cells (approximately 10^6 per ml) were gently shaken with the test suspensions in a shaking water bath at 37°C for 2 h. At the end of this period, [^{14}C]aminopyrine ([^{14}C]AP), histamine (10^{-4} M), and the phosphodiesterase inhibitor isobutyl methylxanthine (10^{-4} M) were added. Appropriate controls with no histamine or isobutyl methylxanthine (basal) and 2,4-dinitrophenol to kill the cells (nonspecific binding) were included. After incubation, triplicate samples were centrifuged in 1.5-ml Microfuge tubes. The tips were cut off, and cell pellets were digested with sodium hydroxide. After neutralization, aqueous scintillation cocktail was added, and samples were counted. A ratio of [^{14}C]AP accumulated in stimulated parietal cells relative to that in treated cells, minus basal uptake medium after subtraction of the radioactivity nonspecifically bound to the 2,4-dinitrophenol-killed cells, was calculated. To compare the effect of each bacterial preparation, results are expressed as a percent inhibition of ^{14}C accumulation in those cells incubated with a bacterial suspension compared with stimulated parietal cells. Data were normalized to percents because day-to-day variations of ratios with cells obtained from different animals made direct comparison impractical. Each cell preparation used included 2,4-dinitrophenol, basal, and stimulated samples. All test samples were tested blind by using coded preparations of bacteria. Student's *t* test was used to assess statistical significance.

Electron microscopy. Small pieces of mouse gastric mucosa, infected with *H. felis*, were fixed in glutaraldehyde, thin sectioned, stained with osmium tetroxide, and examined with an electron microscope as previously described (11).

RESULTS

As previously reported, *H. pylori* (isolate H.win) inhibited the accumulation of [^{14}C]AP uptake by parietal cells (Fig. 1). Eight other clinical isolates of *H. pylori* have also been tested and have been shown to inhibit acid secretion comparably (data not shown). *H. felis* and an *H. pylori*-like isolate from a rhesus monkey proved to be equally as inhibitory to [^{14}C]AP accumulation as their human counterpart. The inhibition of stimulation by these three species was not statistically different from the unstimulated state; indeed, acid secretion was frequently reduced below basal levels. In contrast, the American Type Culture Collection strain of *H. mustelae* had little effect on the accumulation of [^{14}C]AP. Two other isolates of this organism behaved similarly, whereas two others profoundly inhibited secretion. Two urease-producing control bacteria, *P. vulgaris* and *K. oxytoca*, and one other facultative anaerobe, *E. coli*, along with *C. jejuni* reduced [^{14}C]AP accumulation by less than 25%, an amount insignificantly less than the stimulated value. It is likely that there were some variations in bacterial numbers of these control organisms, as we relied on an optical density at 600 nm of 1 for standardizing comparisons, rather than absolute counts of CFU. Furthermore, some growth of the control bacteria may have occurred during the 2-h incubation period, when whole bacteria were under study. The fastidious nature of *Helicobacter* species makes it unlikely that the bacteria replicated during the experiment in which high oxygen concentrations were used.

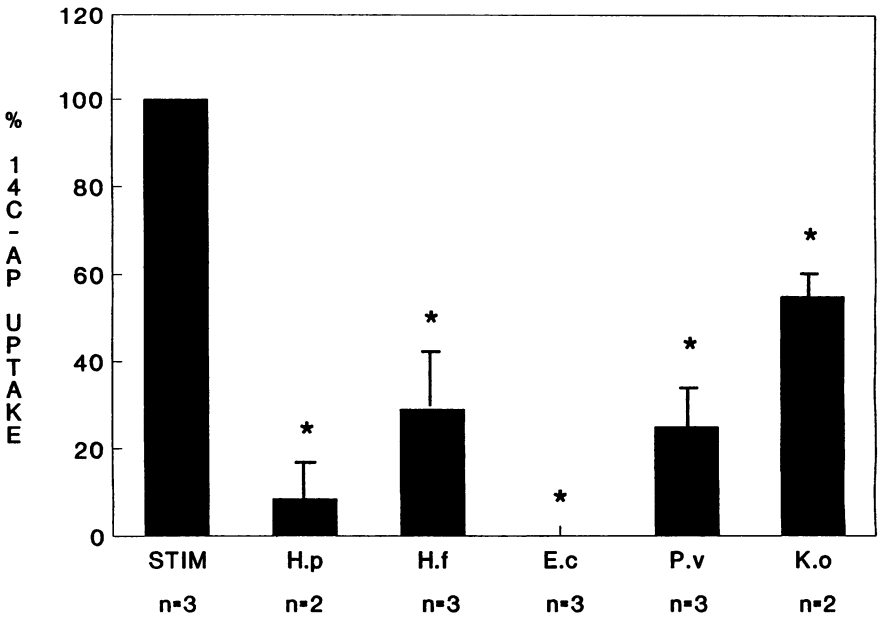

FIG. 2. Effect of cell-free sonicates of bacteria on the uptake of [^{14}C]AP by rabbit parietal cells. K.o, *K. oxytoca*. Other abbreviations and symbols are same as for Fig. 1.

The use of bacterial sonicates, instead of whole bacteria (Fig. 2), had little influence on [^{14}C]AP accumulation as far as *H. pylori* and *H. felis* were concerned. However, *P. vulgaris*, *K. oxytoca*, and particularly *E. coli*, when sonicated, all significantly reduced stimulated [^{14}C]AP accumulation to lower levels than the intact organism. Given the differences between the uses of whole and sonicated bacteria, we heated the whole bacteria to 60°C for 30 min (Fig. 3). This revealed some heat lability for *H. pylori*, increasing uptake of [^{14}C]AP to 46%, and for *H. felis*, increasing uptake to 52%. Comparable reductions were seen with the control organisms. There was no significant difference between the amounts of inhibition by any of the heated organisms.

We have further explored the effect of pronase on the acid inhibitor (Fig. 4). The addition of histamine and isobutyl methylxanthine stimulated acid secretion substantially. However, when *H. pylori* was treated with pronase, little reduction in stimulation was seen, showing that the inhibitor

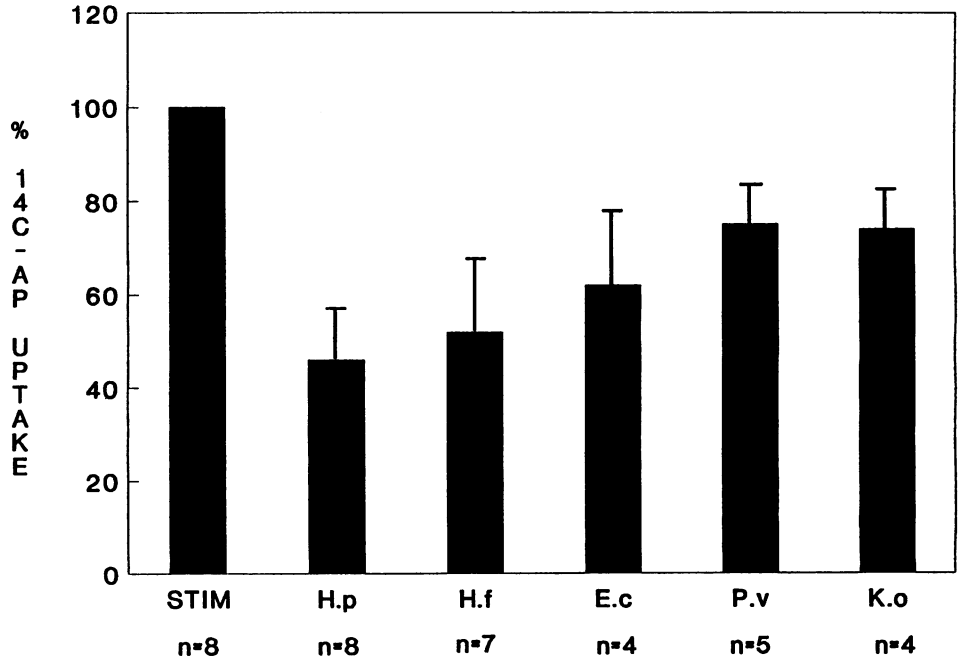

FIG. 3. Effect of prior heating of bacteria to 60°C for 30 min on uptake of [^{14}C]AP by rabbit parietal cells. Abbreviations and symbols are same as for Fig. 2.

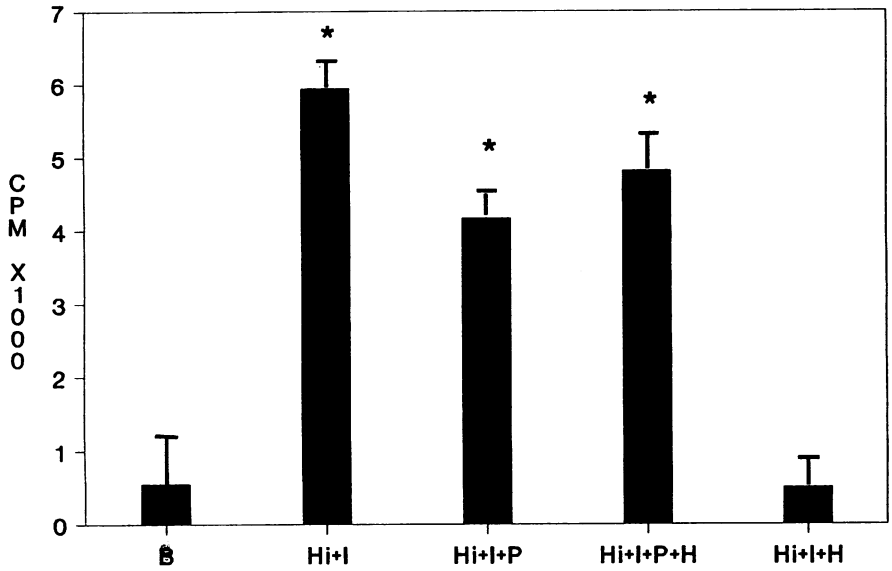

FIG. 4. Effect of pronase treatment (50 μg/ml for 30 min) on the acid inhibitor from *H. pylori* as measured by the change in uptake of [^{14}C]AP by rabbit parietal cells (in counts per minute). Abbreviations: B, basal; H, *H. pylori* sonicate; Hi, histamine (10^{-4} M); I, isobutylmethyl xanthine (10^{-4} M); P, pronase. Standard errors are represented by vertical lines from the mean. Asterisks represent statistical significance.

is protease sensitive. Pronase alone interferes little in the assay.

Figure 5 demonstrates the presence of *H. felis* within a murine parietal cell. At low power (Fig. 5A), multiple organisms can be seen. In Fig. 5B, one organism is seen in close apposition to the cell membrane, with neither appearing damaged.

DISCUSSION

We have previously shown that *H. pylori* produces a factor, probably a protein, that inhibits acid secretion from rabbit parietal cells (3). Data reported here show that this property is shared by other *Helicobacter* species but by not all isolates. *H. felis*, *H. pylori*, and some but not all isolates of *H. mustelae* are able to switch off acid secretion by the rabbit parietal cell. This antisecretory activity of gastric colonizers suggests that this is an evolved mechanism that allows the organism to survive in the stomach early in the natural history of infection. Data described above show that pronase destroys the *H. pylori* inhibitor and that the pronase alone has little effect on the assay. This result further suggests that the factor at least contains a protein component. The antisecretory activity of *H. pylori* provides an explanation for the relatively transient hypochlorhydria that has been reported to be associated with acute infection in humans (17) as well as with experimental infection with *H. mustelae* in ferrets (7).

Despite the appeal of the above hypothesis, there are a number of anomalies that need explanation before it can be claimed that antisecretory activity is a major factor in gastric colonization. Though substantial numbers of humans are infected with *H. pylori*, the majority secrete approximately normal amounts of acid. However, it does appear, at least in some cases, that in the early stages of infection the gastric juices were at a neutral pH. The best example is the study of a volunteer reported by Morris and Nicholson (14). Given that hypochlorhydria is an early event, it may be that it is important in allowing the organism to initially colonize. The physiological effects of early colonization by *H. felis* in animals have yet to be determined. In an experiment with germ-free dogs, one of five *H. pylori*-colonized animals and two of five *H. felis*-infected animals, orally dosed 30 days previously, had pHs of more than 6.0. Small numbers and lack of sufficient control dogs make it difficult to evaluate the importance of this result (16).

If *H. pylori*, *H. felis*, *H. mustelae*, and the rhesus monkey isolate switch off acid secretion in vitro, then why are not persistently colonized humans and animals hypochlorhydric? Several explanations are worthy of consideration. First, different proportions of gastric epithelium are colonized at different stages of infection. Examination of the data for the limited number of early cases available suggests that in the early stages of infection with *H. pylori* there are more organisms present, and it may be that more of the surface of the stomach is infected. Thus there is sufficient acid inhibitor produced to inhibit all or nearly all parietal cells. However, the degree of hypochlorhydria in association with *H. pylori* is profound. This raises the issue of whether a single organism can produce sufficient acid inhibition to have an effect in its immediate proximity or whether it produces a more global effect. It may be that cultured *Helicobacter* species are in a down-regulated state, i.e., under conditions of culture or chronic infection, the bacterium produces a minimal amount of acid inhibitor that is insufficient to decrease overall acid secretion but which may locally reduce secretion. Previous data have shown that very little acid inhibitor is secreted in vitro into media (3). However, acute infection may be associated with up-regulation of acid inhibitor secretion to a degree that enough acid inhibitor is produced to cause complete acid inhibition. This inhibition persists until such time that local environmental factors again cause down-regulation. This theory provides a better explanation than one based on numbers of bacteria, since increasing numbers of bacteria would be likely to give a spectrum of pH values, whereas the pH is usually near

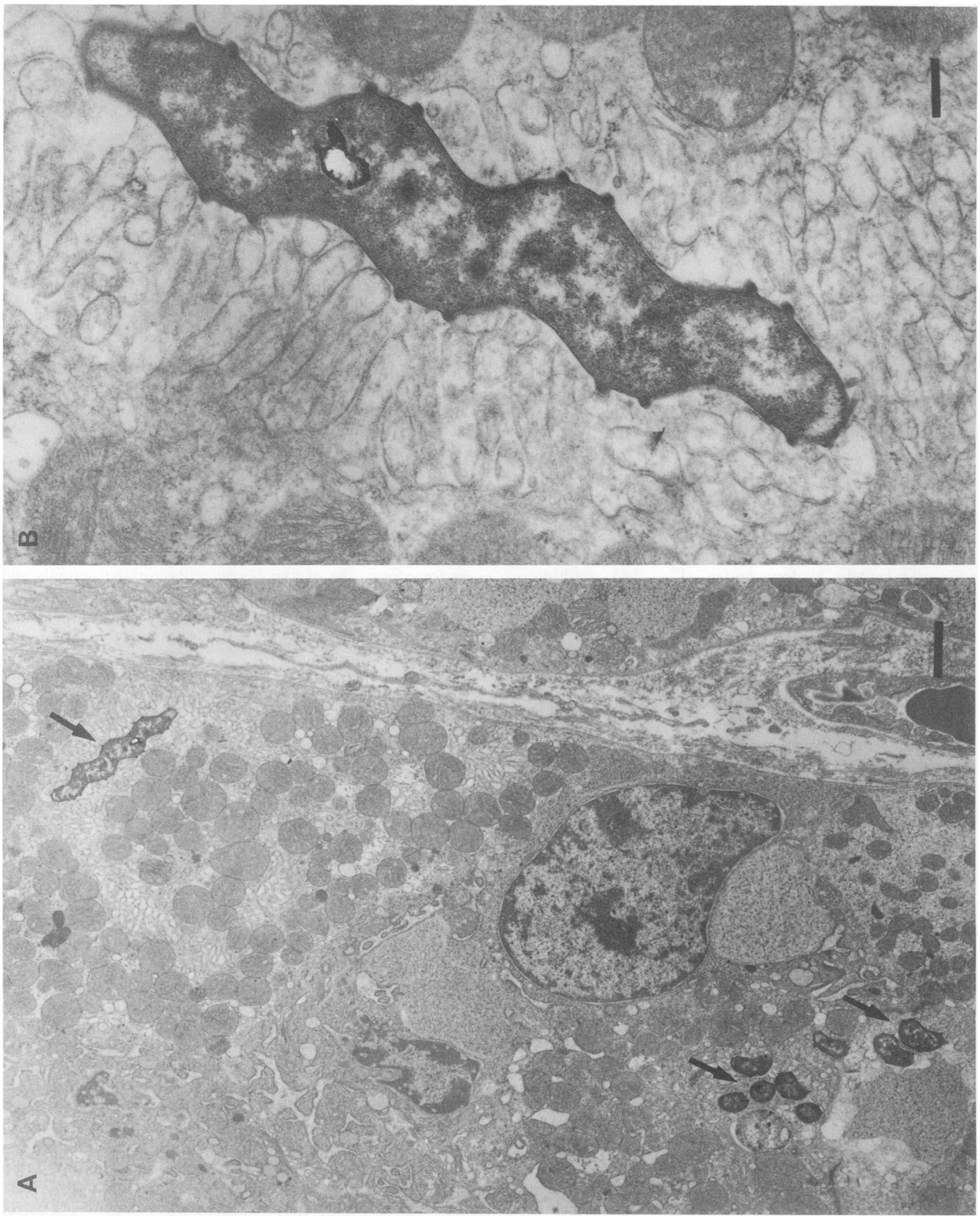

FIG. 5. *H. felis* within the canalicular system of parietal cells of a germ-free mouse. Bar in panel A = 1 μm; bar in panel B = 0.2 μm.

neutral or very acid. Examples of this type of environmental signal transduction from pathogenic bacteria are now well described (13).

The characteristic morphology of the organism *H. felis*, revealed by electron microscopy, makes identification of it and similar bacteria easy. The photomicrograph (Fig. 5) shows bacteria of this morphology in parietal cells of a mouse. Similar observations have been made in cells of dogs (9) and humans (4). The unexpectedly common occurrence of these organisms within the low pH environment of the parietal cell canaliculus may be explained by the secretion of an acid-inhibitory protein resulting in an increase in pH of the canaliculus, thus allowing bacteria safe access to the canalicular system. This explanation is more attractive than that of the urease converting urea to ammonia, which in turn provides a local increase in pH. There is some evidence that the ammonia causes cell damage (10). Though there is no evidence of cell damage in Fig. 5, damaged parietal cells have been observed in dogs infected with gastric spirilla (9).

There is a further anomaly that needs resolution before too much emphasis is placed on the importance of the acid-inhibitory effect. *H. pylori*, *H. felis*, *H. mustelae*, and other gastric spirilla seem to prefer the non-acid-secreting antral mucosa, even though they can heavily colonize the acid-producing fundus (2, 5, 6, 15). If one presumes that the pattern of colonization of the human volunteer, described by Morris and Nicholson (14), is the normal pattern of infection, then this is illustrated. At day 5 postinfection, the antrum was heavily infected and acute inflammation was present. Histologically, the fundic mucosa was normal and no bacteria were observed. In was not until day 8 that the body of the stomach became infected, coincident with the development of hypochlohydria, and that gastritis was observed. This observation appears to argue against a role for antisecretion in very early colonization.

The difference in effect between different isolates of *H. mustelae* suggests a specific effect by the acid inhibitor rather than a nonspecific one mediated by a substance such as lipopolysaccharide. Whether the failure to produce an acid inhibitor by some *H. mustelae* isolates is a genetic deficiency or one of unfavorable cultural conditions remains to be evaluated. As might be expected, the sonication of control bacteria released factors that inhibited the assay specifically or nonspecifically. Heat treatment diminished the effect of both *Helicobacter* species and control organisms and was not a useful discriminant. The [^{14}C]AP assay was designed for physiological and phamacological studies. We have tested crude bacterial preparations, so it is not unexpected that there are variations among different bacteria. We have used the assay as a screening tool that should be progressively more useful as the inhibitor is purified and the variables are reduced.

Despite the unresolved issues, the ability of these gastric bacteria to manufacture an antisecretory protein is likely to be more than coincidence and to be important to these organisms' ability to colonize the gastric mucosa as well as parietal cells. Purification of this putative protein is required before it will be possible to say whether there is one protein that is specific to *Helicobacter* species or whether the protein is found in other bacteria as well.

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